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the American Type Culture Collection under Designation No. HB-12467.

REMARKS

Claims 81-91 are pending. Claims 81-83 have been amended to introduce certain format changes. Support for these amendments may be found at page 32, lines 9-10, of the specification. Applicants submit that these amendments raise no issue of new matter. Thus, claims 81-91 are still pending and under examination.

Pursuant to the requirements of 37 C.F.R. §1.121, applicants annex hereto as **Exhibits A** and **B** a copy of the amended paragraphs of the specification and the amended claims, respectively, marked up to show the changes made herein relative to the previous version thereof.

In view of the arguments set forth below, applicants maintain that the Examiner's rejections made in the November 19, 2002 Office Action have been overcome, and respectfully request that the Examiner reconsider and withdraw same.

Formalities

The Examiner stated that certain drawing corrections are required. In response, applicants note that corrected formal drawings will be submitted upon allowance of the claims, pursuant to the Draftsperson's requirements.

Rejections Under 35 U.S.C. §112, First Paragraph

The Examiner rejected claims 81-91 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to

reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner alleges that the specification lacks support for the term "EP-hCG."

In response to the Examiner's rejection, but without conceding the correctness thereof, applicants note that the amended claims recite "EPMI-hCG" to indicate early pregnancy associated molecular isoform of hCG detected by the B152 antibody. Support for this amendment may be found in the specification at page 32, lines 9-10, page 33, lines 9-10, and at page 34, lines 1-3.

The Examiner also rejected claims 81, 82, and 87-91 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention.

Applicants understand the Examiner's rejection to be based upon her assertion that "[t]he antibody itself is required [to demonstrate possession of the claimed invention]." The Examiner relied, in part, upon *Amgen* to support this position. *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1206 (Fed. Cir. 1991).

Applicants respectfully disagree with the Examiner's position, and maintain that the Examiner has misapplied *Amgen* in support of this rejection.

According to M.P.E.P. §2163.02, "[p]ossession may be shown in a variety of ways including … describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention." The M.P.E.P. also cites *Amgen* for the proposition that "one must define a compound by 'whatever characteristics sufficiently distinguish it.'"

The antibodies of the instant methods can be distinguished from other antibodies by their relative binding affinities for hCG isoforms. For example, the B151 and B152 antibodies are distinguished on the basis of their affinities for C5 chorio hCG and nicked hCG, as taught in the specification at page 76, lines 22-32 and following on page 77, lines 1-8, and in Figure 12. Importantly, no knowledge of an antibody's structure is needed to determine its affinity for a particular antigen. Instead, affinity can be determined by well known binding assays, for example, that shown on pages 336-337 of Creighton (1984), attached hereto as **Exhibit C**.

The Examiner's assertion that a "detailed structure" of an antibody is required to demonstrate possession is not supported by *Amgen*. Applicants maintain that the binding properties of the working examples provided in the specification are sufficient to distinguish those properties for the antibodies used in the claimed methods.

Applicants also note that the specification describes how to obtain the antibodies of the claimed methods, for example, at page 85, lines 1-28. Applicants maintain that the teachings of the instant specification, combined with the high level of skill in the art of antibody production, demonstrate that

applicants were in possession of the antibodies used in the instant methods.

In her rejection of claims 81, 82, and 87-91 under 35 U.S.C. §112, first paragraph, the Examiner also relied upon the assertion that "due to lack of written description of the structure or identity of the [EPMI-hCG] molecule, one cannot generate other antibodies to the specific [EPMI-hCG] molecule of the claimed invention."

The Examiner's assertion is without merit. Applicants maintain that possession of EPMI-hCG is not required to practice the claimed invention because antibodies that bind to EPMI-hCG can be generated using other immunogens that were known in the art. For example, as taught in the specification at page 40 lines 4-7, the immunogen used to elicit the B152 antibody itself was choriocarcinoma-derived hCG, designated "C5." The source, purification, and structure of the C5 immunogen are taught in Kardana et al. (1991), attached hereto as **Exhibit 7**, and in Elliot et al. (1997), attached hereto as **Exhibit 6**. The specification further teaches at page 40, lines 20-26, that JAR choriocarcinoma cells produce hCG recognized by the B152 antibody. Applicants maintain that either the C5 or JAR-derived hCG could be used to generate antibodies that bind to EPMI-hCG. In view of the teachings of the specification and the knowledge of choriocarcinoma-derived hCG in the prior art, applicants maintain that it is not necessary for the instant specification to further describe EPMI-hCG.

In summary, applicants maintain that they have demonstrated possession of the antibodies used in the claimed methods, and of the claimed methods themselves, in satisfaction of the written description requirement.

In view of these remarks, applicants maintain that claims 81, 82, and 87-91 satisfy the requirements of 35 U.S.C. §112, first paragraph, and respectfully request that the Examiner withdraw her rejection thereto.

Rejection Under 35 U.S.C. §112, Second Paragraph

The Examiner rejected claims 81-91 under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Specifically, the Examiner alleged that claims 81 and 82 are vague and indefinite due to the recitation of the term "EP-hCG".

In response, applicants respectfully traverse. Applicants note that amended claims 81 and 82 recite the term "EPMI-hCG", meaning "early pregnancy-associated molecular isoform of hCG." Support for this term is found in the specification at page 32.

In view of the above remarks, applicants maintain that claims 81-91 satisfy the requirements of 35 U.S.C. §112, second paragraph.

Information Disclosure Statement

In accordance with their duty of disclosure under 37 C.F.R. §1.56, applicants request that the following disclosures be made of record in the above-identified application pursuant to 37 C.F.R. §1.97(c). These references are also listed on the attached Form PTO-1449 (**Exhibit D**). Copies of items 1-7 are attached hereto as **Exhibits 1-7**, respectively.

1. U.S. Publication No. 2003/0022381, Pandian et al. published January 30, 2003 **(Exhibit 1)**;

2. Ehrlich P.H. and Moyle W.R. (1983) "Cooperative immunoassays: ultrasensitive assays with mixed monoclonal antibodies," Science 221:279-81 **(Exhibit 2)**;

3. Ehrlich P.H. et al. (1982) "Mixing two monoclonal antibodies yields enhanced affinity for antigen," J. Immunol. 128(6):2709-13 **(Exhibit 3)**;

4. Ehrlich P.H. et al. (1985) "Characterization and relative orientation of epitopes for monoclonal antibodies and antisera to human chorionic gonadotropin," Am. J. Reprod. Immunol. Microbiol. 8(2):48-54 **(Exhibit 4)**;

5. Ehrlich P.H. et al. (1985) "Monoclonal antibodies to gonadotropin subunits," Methods Enzymol. 109:638-55 **(Exhibit 5)**;

6. Elliott, M.M. et al. (1997) "Carbohydrate and peptide structure of the alpha and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma," Endocrine 7(1):15-32 **(Exhibit 6)**; and

7. Kardana, A. et al. (1991) "The heterogeneity of human chorionic gonadotropin (hCG). I. Characterization of peptide heterogeneity in 13 individual preparations of hCG," Endocrinology 129(3):1541-50 **(Exhibit 7)**.

Summary

In view of the amendments and remarks made herein, applicants maintain that the claims pending in this application are in condition for allowance. Accordingly, allowance is respectfully requested.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee, other than the enclosed fee, is deemed necessary in connection with the filing of this Amendment. However, if any fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to:
Assistant Commissioner for Patents
Washington, D.C. 20231

3/19/03

Alan J. Morrison — Date
Reg. No. 37,399

Marked-up version of amended paragraphs of the specification

The paragraph beginning at page 12, line 17:

Immunoassay profiles of fractions from SUPEROSE 12™ (Pharmacia) ~~SUPEROSE 12~~ column chromatography of a pooled urine concentrate from pregnant women.

The paragraph beginning at page 76, line 12:

Characteristics of antibodies

A variety of hCG isoforms were employed to characterize the new antibodies described in this report and the nomenclature and characteristics of each of the reagents employed is summarized in ~~Table 1~~Figure 11. The carbohydrate groups in these hCG isoforms as well as the percent nicking were analyzed in an earlier study (26) and are directly relevant for defining the nature of these new antibodies in this report.

The paragraph beginning at page 76, line 22:

Two antibodies designated B151 and B152 were selected by the use of radiolabeled hCG isoforms, chorioCG C5 and pregnancy hCG CR_127. Each displayed preferential binding to C5 as compared to CR 127 since this was the selection criterion. However, upon performing liquid phase immunoassays and calculating affinity constants, it was clear that these two antibodies were very different in specificity (Figure ~~16~~12). It was found that antibody B151 had one order of magnitude higher affinity both for C5, which is nicked and hyperglycosylated choriocarcinoma

hCG, and for CR_127 hCGn (813) as compared to CR 127 hCG or nick-free CR 127(814) (see Figure ~~15~~ 11 for reagent descriptions). B151 was clearly an antibody with a strong preference for binding to various forms of nicked hCG. Antibody B152 was different in that although it displayed one order of magnitude preference for C5 hCG over CR_127 hCG, it recognized the nicked and non-nicked forms of CR 127 hCG, hCG derived from normal pregnancies, to an equal extent.

The paragraph beginning at page 77, line 10:

Liquid Phase Assays

Figure ~~1~~ 8 shows potency comparisons of liquid phase immunoassays of both B151 and B152 antibodies comparing competitors: 1. standard CR 127 pregnancy hCG (which has a 20% content of nicked hCG); 2. C5 chorio CG (100% nicked and hyperglycosylated); 3. 813, nicked CG made from CR 127 by purification, and 4. 814, non-nicked hCG derived from CR 127. The labeled ligand was C5 chorio CG. It is apparent that B151 (Fig ~~1A~~8A) shows a preference for nicked forms of hCG. C5 chorio-CG or 813 hCGn bind with similar affinities. The slightly lower potency of 813 hCGn may be ascribed to its 20% contamination with non-nicked hCG. B152 only shows a preference to C5, the hyperglycosylated chorio CG (Fig ~~1B~~8B)~~—~~. 813 hCGn is no more potent a competitor than nick-free 814 hCG.

The paragraph beginning at page 77, line 26:

Immunometric Two Site Assays

A variety of two site antibody formats were tested. Figure ~~17~~ 13 displays these results. It is apparent that B151 cannot bind simultaneously with antibodies (designated by us as site IV) (27) to the beta subunit and beta subunit core (B201 and B204) nor with antibodies directed towards the determinant which exists in heterodimeric hCG as represented by antibody B109 (site III, to which A109 also belongs) (27). In contrast, a general beta antibody which binds to the most common and potent hCG antigenic site previously designated by us as site II (B108 or B207) binds well simultaneously with both B151 and B152 antibodies. B152 binds simultaneously to all antibodies tested except for those to the beta COOH-terminal region (CTP) (28) in contrast to B151 which binds well to CTP antibodies. B151 may represent a newly revealed hCG epitope which only exists on nicked hCG as reported in this manuscript.

The paragraph beginning on page 79, line 1:

Characteristics of the B152-B207-I^{125} radioimmunometric two-site assay

In order to better understand what this assay is measuring, we compared the relative binding potencies of a series of isoforms of hCG shown in ~~figure~~ Figure ~~2~~ 9 (also see methods):1. C5, choriocarcinoma hCG 2. 814, non-nicked hCG. 3. 813, nicked hCG (80% nicked). 4. M4 mole-derived hCG, 98% nicked hCG with negligible hyperglycosylation. 5. MIA hCG, non-nicked and not significantly

hyperglycosylated but missing 80% of the hCG beta COOH-terminus. The B152 two-site assay prefers to bind to C5, its immunogen, but shows nearly equal recognition of both 813 and 814, nicked and non-nicked hCG of normal pregnancy. This confirms that B152 does not display significant preference for the nicked form of hCG but rather for the form with carbohydrate differences. This is also confirmed by the potency of M4 which is also 100% nicked as is C5 but is not hyperglycosylated and displays a potency similar to CR_127 hCG whether nicked or non-nicked. MIA is the least potent ligand and is the only one missing most of its beta COOH-terminal peptide confirming the role of this region in the B152 epitope.

The paragraph beginning on page 79, line 24:

In order to further explore the nature of the B152 binding site, a commercial~~l~~ly available peroxidase-labeled general hCGβ antibody (4001) was employed as a detection antibody in a two-site enzyme immunometric system. Eight different hCG forms were evaluated in this system illustrated in ~~figure~~ Figure ~~14~~10. Results are analyzed in terms of relative immunopotency (based on the slope of the regression line) in ~~figure~~ Figure ~~18~~14. Linear regression correlation analysis was performed to compare the relationship of the immunopotencies of preparations 814, C5, M4, C7 and P8 one at a time with the carbohydrate differences (Figure ~~15~~11) as well as nicking differences among the heterodimeric isoforms of hCG. The correlation results for each comparison are as follows: 1. Tetrasaccharide O-linked core: R^2=0.9147 P=0.0108, significant; 2. Trianntennary branched moieties N-linked

on β: R^2=0.8853 P=0.0171, significant; 3. Sialic acid O-linked: R^2=0.3062 P=0.3332, not significant; 4. Sialic acid N-linked on β: R^2=0.2289 P=0.4149, not significant; 5. Percent nicking in β subunit: R^2=0.0984 P=0.6072, not significant.

Marked-up version of amended claims:

81. (amended): A method for detecting a gestational trophoblast malignancy in a subject who is either pregnant or suspected of being pregnant, comprising the steps of:

(a) (i) contacting a first portion of a urine sample from the subject with an antibody which binds to EPMI-hCG under conditions permitting the formation of a complex between the antibody and any EPMI-hCG present in the sample; and

(ii) measuring the amount of any complex formed, so as to thereby determine the amount of EPMI-hCG in the sample;

(b) (i) contacting a second portion of the urine sample from the subject with an antibody which binds to intact hCG under conditions permitting the formation of a complex between the antibody and any intact hCG present in the sample; and

(ii) measuring the amount of any complex formed, so as to thereby determine the amount of intact hCG in the sample, with the proviso that steps (a) and (b) can be performed in any order;

(c) determining the ratio of EPMI-hCG to intact hCG in the sample from the measurements performed in (a)(ii) and (b)(ii); and

(d) repeating steps (a) through (c) at least once over a suitable time period, wherein a ratio of EPMI-hCG to intact hCG greater than 1.0 occurring

over such time period indicates the presence of a gestational trophoblast malignancy.

82. (amended): A method for detecting a gestational trophoblast malignancy in a subject who is either pregnant or suspected of being pregnant, comprising the steps of:

(a) (i) contacting a first portion of a urine sample from the subject with a first antibody which binds to EPMI-hCG under conditions permitting the binding of the first antibody with any EPMI-hCG present in the sample, wherein the first antibody is bound to a solid support;

(ii) removing any unbound sample from the solid support;

(iii) contacting the solid support with a second antibody which binds to bound EPMI-hCG under conditions permitting the binding of the second antibody to bound EPMI-hCG; and

(iv) measuring the amount of the second antibody bound to the bound EPMI-hCG, so as to thereby determine the amount of EPMI-hCG in the sample;

(b) (i) contacting a second portion of the urine sample with a third antibody which binds to intact hCG under conditions permitting the binding of the third antibody with any intact hCG present in the sample, wherein the third antibody is bound to a solid support;

(ii) removing any unbound sample from the solid support;

(iii) contacting the solid support with a fourth antibody which binds to bound intact hCG under conditions permitting the binding of the fourth antibody to bound intact hCG; and

(iv) measuring the amount of the fourth antibody bound to the bound intact hCG, so as to thereby determine the amount of intact hCG in the sample, with the proviso that steps (a) and (b) can be performed in any order;

(c) determining the ratio of EPMI-hCG to intact hCG in the sample from the measurements performed in (a)(iv) and (b)(iv); and

(d) repeating steps (a) through (c) at least once over a suitable time period, wherein a ratio of EPMI-hCG to intact hCG greater than 1.0 occurring over such time period indicates the presence of a gestational trophoblast malignancy.

83. (amended): The method of claim 81 or 82, wherein the antibody which binds to EPMI-hCG is B152, deposited with the American Type Culture Collection under Designation No. HB-12467.

PROTEINS

Structures and Molecular Principles

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[redacted]

8

INTERACTIONS WITH OTHER MOLECULES

The biological functions of proteins almost invariably depend upon their direct, physical interaction with other molecules. Virtually every substance with which a cell comes into contact is recognized and bound by some protein, and every small molecule within a cell was first bound by the enzyme that produced it or by the receptor on the cell surface that enabled it to be taken up. Proteins may bind very tightly and specifically to other proteins, generating large complexes; to nucleic acids, especially when controlling their replication and expression; to polysaccharides, especially important being those on the surfaces of cell membranes; and to lipids, often becoming incorporated within membranes. Every aspect of the structure, growth, and replication of an organism is dependent upon such interactions.

Proteins are generally classified according to the purpose and consequences of binding—for example, structural proteins, enzymes, repressors, lectins, toxins, immunoglobulins, hormones, receptors, membrane transport proteins, and proteins of motility. The physical principles of the interactions are similar in all these cases and are the subject of this chapter. (Systems involving chemical changes, as in enzyme catalysis, active transport, and mechanical movement, are left to the next chapter.) The following discussion focuses on the protein; whatever molecule it interacts with, even if it is another protein, is designated the **ligand.**

This chapter emphasizes reversible interactions, in which there is always a significant concentration of free ligand in equilibrium with the complex, or where the protein is normally found in both the free and liganded forms. Accordingly, stable supramolecular assemblies such as viruses and ribosomes, where the complex is the normal structural unit, are

not considered here; likewise, little attention is given to interactions with normal components of the solvent, such as water molecules and hydrogen ions in the case of water-soluble proteins, and lipids in the case of membrane proteins. These interactions have been described earlier and are not fundamentally different, but they occur at many sites on the protein and are generally weak, occurring only because the solvent molecules are present in such high concentrations.

The interactions described here are distinguished by being specific for the appropriate ligand and occurring at a very limited number of sites on the protein, most often just one per polypeptide chain, with only a very limited number of different binding sites. The specificity of binding—that is, the discrimination between even closely related ligands—is determined by their relative binding affinities, which are discussed first. Next, the molecular structures of protein–ligand complexes are described briefly, followed by discussion of the molecular basis of the binding affinity and specificity. The final topic is the consequences of binding for the structure of the protein and how binding sites within a protein interact with each other—that is, the phenomenon of allostery.

ENERGETICS AND DYNAMICS OF BINDING

Binding Affinities

The affinity between a protein (P) and a ligand (A) is measured simply by the association constant, K_a, for the binding reaction at equilibrium:

$$P + A \underset{}{\overset{K_a}{\rightleftharpoons}} P \cdot A \tag{8–1}$$

$$K_a = \frac{[P \cdot A]}{[P][A]} \tag{8–2}$$

All species are presumed to be present at sufficiently low concentration for thermodynamic ideality to apply. K_a is a constant under a given set of conditions and is measured experimentally by the dependence of binding on the free ligand concentration. Several commonly used graphic methods of analyzing binding data are illustrated at Figure 8–1. The ratio of bound to free protein should be directly proportional to the free ligand concentration:

$$\frac{[P \cdot A]}{[P]} = K_a[A] \tag{8–3}$$

An experimentally more useful measure of binding is the fraction (y) of protein molecules with bound ligand:

$$y = \frac{[P \cdot A]}{[P] + [P \cdot A]} = \frac{K_a[A]}{1 + K_a[A]} \tag{8–4}$$

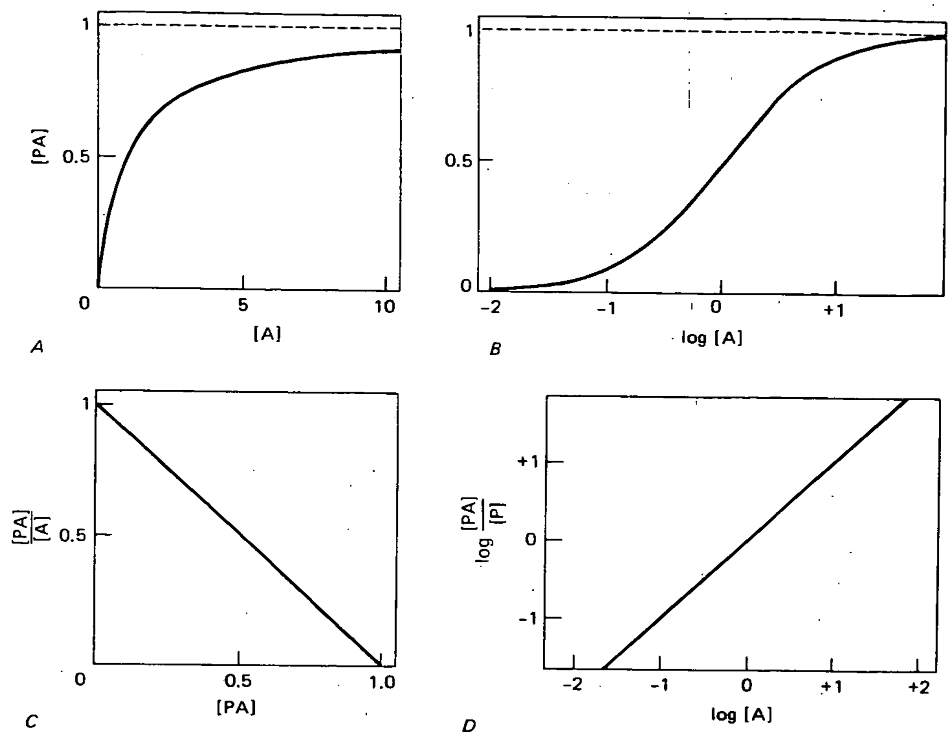

Figure 8–1
Some common methods of plotting binding data, using theoretical curves for the simple binding reaction $P + A \rightleftharpoons P \cdot A$. The concentration of free ligand is expressed relative to its dissociation constant, which is that concentration which gives half maximal binding. The concentrations of free and liganded forms of the protein are relative to the concentration of total protein.

The normal hyperbolic relationship between binding and free ligand concentration is illustrated in A, *demonstrating that a ligand concentration 9-fold greater than its dissociation constant produces only 90 per cent of maximal binding (indicated by the dashed line); 99-fold greater is required for 99 per cent saturation. The large range of free ligand concentrations required for a complete binding curve is emphasized when a logarithmic scale is used, as in* B.

C, *A Scatchard plot. The negative slope gives the value of the association constant (the reciprocal of the dissociation constant). The horizontal intercept gives the extrapolated extent of the maximal binding.*

D, *A Hill plot. An accurate value for the maximum binding is required for this plot, as both the liganded and free protein concentrations are required. The value of the dissociation constant is given by the value of the free ligand concentration where the vertical axis is zero, i.e., at half-maximal binding. This plot is used primarily for analyzing cooperative binding (see Figure 8–3).*

The greater the value of K_a, the greater the affinity. However, the value of K_a has units of (concentration)$^{-1}$, and it is often intuitively easier to consider the dissociation constant, K_d; it is simply the reciprocal of K_a and has units of concentration. With concentrations of free ligand below

K_d, little binding occurs. With a concentration equal to K_d, half the protein molecules have ligand bound. An occupancy of 90 per cent requires a nine times' greater concentration of free ligand, whereas 99 per cent occupancy requires that it be 99 times K_d.

Specific binding of one ligand, and not another, is dependent upon their having different affinities. If two ligands are present at a concentration of 10^{-5} M, but have different values of K_d—say, 10^{-3} M and 10^{-6} M—only the latter will be bound significantly. If both are present at higher concentrations, say, 10^{-2} M, specific binding will still be possible, both would be bound to their individual sites. However, if they compete for the same site:

$$\mathrm{P \cdot A} \underset{K_d^{\mathrm{A}}}{\overset{\mathrm{A}}{\rightleftarrows}} \mathrm{P} \underset{K_d^{\mathrm{B}}}{\overset{\mathrm{B}}{\rightleftarrows}} \mathrm{P \cdot B} \tag{8–5}$$

$$\mathrm{P} = \frac{[\mathrm{P \cdot A}]\, K_d^{\mathrm{A}}}{[\mathrm{A}]} = \frac{[\mathrm{P \cdot B}]\, K_d^{\mathrm{B}}}{[\mathrm{B}]} \tag{8–6}$$

$$\frac{[\mathrm{P \cdot A}]}{[\mathrm{P \cdot B}]} = \frac{K_d^{\mathrm{B}}}{K_d^{\mathrm{A}}} \frac{[\mathrm{A}]}{[\mathrm{B}]} \tag{8–7}$$

In this case, that ligand with the higher affinity would be bound to a correspondingly greater extent.

The energetics of binding are often expressed by the Gibbs free energy of binding, $\Delta G°$:

$$\Delta G° = -RT \ln K_a = RT \ln K_d \tag{8–8}$$

However, it must be kept in mind that K_a and K_d have units of concentration and that the value of $\Delta G°$ will depend upon which units are used, i.e., the standard state. If the units are moles/liter, the standard state is 1 M, and the calculated value of $\Delta G°$ will apply only under the rather arbitrary situation when the concentration of free ligand is 1 M. In many instances a "unitary" free energy of binding is used as a measure of the intrinsic affinity: the free energy that would occur with a hypothetical ligand at a concentration of 55 M, the normal concentration of water. However, this parameter is not of any special significance, except when the ligand is water, and it does not represent the free energy of interaction that would occur in a unimolecular interaction (see pp. 360–366).

The energetics of binding are defined more explicitly as the difference in free energies of the free and bound protein, $\Delta G_b°$:

$$\Delta G_b° = -RT \ln (K_a[\mathrm{A}]) = RT \ln \left(\frac{[\mathrm{A}]}{K_d}\right) \tag{8–9}$$

where the concentration of free ligand must be specified.

As described later, it is currently impossible to rationalize the value of K_a or K_d, but relative affinities for two related ligands (e.g., A and B) are more easily analyzed, because the ratio is dimensionless:

$$\Delta(\Delta G^\circ)_{A-B} = -RT \ln \frac{K_a{}^A}{K_a{}^B} = +RT \ln \frac{K_d{}^A}{K_d{}^B} \qquad (8\text{–}10)$$

The observed affinities of proteins for ligands vary, ranging from very high values, where dissociation is immeasurably small, to very low values, where the concentration of free ligand required for binding is so great as to cast doubt upon the nature of its effect upon the protein. Whether or not a given affinity is significant depends upon the concentration of ligand the protein is likely to encounter; no other generalizations are possible.

Where the affinity is very high, the protein is likely to be found and isolated as the complex; if such a ligand is relatively small, it is designated a **prosthetic group.** Examples are the heme groups of the globins and cytochromes, some coenzymes, and metal ions that are integral parts of the protein structure. With lower, moderate affinities, ligands originally bound are likely to be lost during isolation of the protein, unless they are added to the protein solution.

Ligand binding is quite simple in dilute solution, but proteins often function in extremely concentrated aqueous solutions, as in the cell cytosol. For example, the interior of the red blood cell is about 35 per cent by weight hemoglobin. Consequently, such solutions are very nonideal, and the pertinent equilibria must be expressed in terms of thermodynamic activities of the species, which can be very different from their concentrations. Even though a particular protein might not be present at high concentration, the presence of other molecules rather than water in the environment can lead to substantial excluded volume effects. These will favor any conformational or binding reaction that leads to a more spherical shape; consequently, binding of a ligand is often considerably greater than might otherwise be expected. It is possible that all proteins in the cytosol normally exist bound to each other, to membranes, to cytoskeleton, or to some other organized structure.

Rates of Binding and Dissociation

$$P + A \underset{k_d}{\overset{k_a}{\rightleftharpoons}} P \cdot A$$

$$K_a = \frac{k_a}{k_d} \qquad (8\text{–}11)$$

The rate constant for binding of a ligand to a protein, k_a, may vary considerably, depending upon their sizes and upon what changes must take place in both upon binding. Nevertheless, many small ligands are found to bind very rapidly, at rates approaching those expected for diffusion control, k_D. This may be estimated from the diffusion coefficients of the protein and ligand, D_P and D_A, respectively, treating them as small spherical molecules that must approach within a distance r_{PA}:

$$k_D = 4\pi\, N_A\, (D_P + D_A)\, r_{PA} \qquad (8\text{–}12)$$